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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES AND PROTEINS

XXXVI*. ORGANIC SOLVENT MODIFIER EFFECTS IN THE SEPARATION OF UNPROTECTED PEPTIDES BY REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

The influence of the organic solvent modifier on peptide and polypeptide retention to octadecylsilica supports has been examined. Over a wide range of volume fractions of the organic solvent modifier, ψ_s, unprotected peptides do not show linear dependencies of their logarithmic capacity factors on the composition of binary hydro-organic solvent eluents. Instead, bimodal plots are observed with minima characteristic of the peptide and the organic solvent. The influence of acidic amine buffers on peptide retention behaviour to silica-bonded C_{18} hydrocarbonaceous stationary phases has also been further investigated. With mobile phases of low water content, it is possible to obtain on these alkylsilica supports, elution orders characteristic of a normal or polar phase elution mode. This irregular retention behaviour has been discussed in terms of the participation of multiple retention processes in the interaction of ionised peptides with chemically-bonded alkyl-silicas. The influence of flow-rate on column efficiencies for peptides separated under reversed-phase conditions has been studied. The results confirm that column efficiencies for small peptides decrease with increasing flow-rate and eluent viscosity. The significance of the dependence of ln k' on ψ_s for the isolation of peptides and polypeptides from biological sources is discussed.

INTRODUCTION

Because of its versatility and convenience, reversed-phase high-performance liquid chromatography (RP-HPLC) with chemically bonded hydrocarbonaceous silica supports has proved phenomenally successful for the separation of peptides[1,2]. One of the special attributes of this technique when compared to other, more conven-

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tional chromatographic methods is the ease with which peptide selectivities can be manipulated by variation of the mobile phase composition. A number of specific mobile phase phenomena, including pH control over peptide ionisation levels, pairing ion complex formation and specific solvation effects can be readily exploited in order to modulate peptide retention and enhance selectivity of the reversed phase chromatographic systems. Although the elution of some very polar peptides from porous microparticulate alkylsilicas can be achieved with purely aqueous eluents, in most cases a hydro-organic solvent combination is required. It is a general experience that the retention of small peptides on reversed-phase silicas decreases as the volume fraction of the organic solvent, ψ_s, in the mobile phase is increased over the range ca. $0 < \psi_s < 0.4$. Under these elution conditions, the retention behaviour of small peptides tends to be regular, that is peptides elute in order of increasing hydrophobicities. However, with more hydrophobic peptides and some small proteins which require mobile phases of higher organic solvent content to affect elution from alkylsilicas, retention order reversals, indicative of a normal-phase separation mode, have been observed[1]. Furthermore, the retention behaviour of polypeptides and proteins is particularly responsive to changes in the water content of the mobile phase. In many cases these larger solutes can only be efficiently chromatographed on chemically bonded alkylsilicas under isocratic conditions which encompass a very narrow range of organic solvent percentages. With mobile phases of high organic solvent content, e.g. when ψ_s is greater than 0.5, it has been noted[3] that the relative selectivity factors for larger peptides separated on octyl- or octadecylsilicas tend to be lower than values obtained with mobile phases of lower solvent percentage. Despite the widespread use of organic solvent modifiers in RP-HPLC separations of peptides, few studies have systematically examined the physico-chemical basis of this modulation of selectivity as the volume fraction of the organic solvent is altered. The purpose of the present study was to compare the elution behaviour of a variety of unprotected peptides using mobile phases of widely differing organic solvent compositions. The results clearly implicate the involvement of multiple retention processes in the interaction of peptides with chemically bonded alkylsilicas.

EXPERIMENTAL

Chemicals and reagents

Acetonitrile, methanol and 2-propanol were all AnalaR or HPLC grade. Water was double distilled and deionised using a Milli-Q system (Millipore, Bedford, MA, U.S.A.). The peptides and polypeptides (Table I) used in this study were purchased from Sigma (St. Louis, MO, U.S.A.), Bachem (Torrance, CA, U.S.A.) or Research Plus Labs. (Denville, NY, U.S.A.). All amino acids except glycine were of the L-configuration. Orthophosphoric acid was obtained from May and Baker (Dagenham, Great Britain). Triethylamine was from BDH (Poole, Great Britain) and purified prior to use[5,6].

Apparatus

The chromatographic data were collected with one (isocratic) or two (gradient) Model M6000A solvent delivery pumps, a M660 solvent programmer, a U6K universal chromatographic injector, a Model M440 fixed wavelength (254 nm) or a

TABLE I

PEPTIDES AND POLYPEPTIDES USED IN THE PRESENT STUDY

The one letter code for the amino acids is used as given by M. O. Dayhoff in *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Silver Spring, MD, U.S.A., 1972.

No.	Peptide	No.	Peptide
1	F	9	RF
2	FF	10	RFA
3	FFF	11	DRVYIHPF (Angiotensin II)
4	FFFF	12	DRVYIHPFHL (Angiotensin I)
5	FFFFF	13	Bovine insulin B chain
6	LY	14	Bovine insulin
7	PY	15	Porcine glucagon
8	YYY		

Model M450 variable-wavelength UV detector, all from Waters Assoc. (Milford, MA, U.S.A.) coupled to a Rikadenki or Omniscribe dual channel recorder. Sample injections were made with Pressure Lok liquid syringes (0–25 μl) from Precision Sampling (Baton Rouge, LA, U.S.A.). The pH measurements were performed with a Radiometer, PHM64 Research pH meter, equipped with a combination glass electrode.

Methods

All chromatograms were carried out at ambient temperatures. Bulk solvents and mobile phases were prepared and degassed by sonication as reported previously[3,4]. This procedure ensures that no preferential evaporation of the organic solvent occurs from mobile phases of high organic solvent content. A single μBondapak C_{18} column (particle diameter 10 μm, 30 × 0.4 cm I.D.) was used throughout this study. The column was equilibrated for at least 30 min (*ca.* 50–100 column volumes) with new mobile phases conditions. Sample sizes varied between 2 and 5 μg of peptide material injected in volumes ranging between 2 and 5 μl. The capacity factors were calculated in the usual way with $NaNO_3$ to calibrate the column. Column efficiencies in terms of theoretical plates (N) were determined under isocratic conditions from the retention time and peak width at the half height and are uncorrected for extra-column zone spreading effects. Apparent plate counts from gradient elution were also calculated in the same manner. The ionic strength and pH of the phosphate buffers were chosen on the basis of criteria established[3,4,7–9] in earlier comparative studies to ensure adequate control over protic equilibria.

RESULTS AND DISCUSSION

The role of the organic solvent modifier in the manipulation of the retention of polar solutes in RP-HPLC has attracted considerable theoretical and experimental attention during the past few years[10–22]. From a practical viewpoint, it is well recognised that both the molecular characteristics and the concentration of the organic solvent in binary hydro-organic solvent eluents have significant influences on the overall chromatographic distribution equilibria established by the solutes. For a

given separation temperature, linear relationships between the logarithmic capacity factors of neutral and anionic solutes and the volume fraction of the organic solvent modifier, ψ_s, in binary hydro-organic solvent eluents are frequently observed. Snyder and coworkers[11,13] have proposed that such linear dependencies of ln k' on ψ_s are representative of regular retention behaviour in RP-HPLC with hydro-organic solvent eluents. Schoenmakers et al.[14], have however argued that ln k' varies quadratically on solvent composition although over the narrow range in k' values of interest in chromatographic optimisation, e.g. $1 < k' < 20$, it has been concluded that linear relationships provide an adequate approximation. Assuming that the retention process in RP-HPLC was essentially determined by solvent effects, Horváth and coworkers[17,19] adapted solvophobic theory to evaluate the role of the eluent in the retention behaviour of neutral and polar solutes on non-polar stationary phases. According to this comprehensive theoretical treatment, the chromatographic process can be viewed as a series of reversible hydrophobic interactions between the solute molecules and the alkyl ligands at the surface of the stationary phase with solute retention governed to a large extent by the bulk surface tension, γ, and the dielectric constant, ε, of the eluent. For water-rich eluents encompassing a narrow range of ψ_s values, e.g. $0.2 < \psi_s < 0.4$, the observed retention behaviour of neutral[20-22] and even some polar solutes including peptides[3,19] has been in good agreement with the regular behaviour expected on the basis of the solvophobic effect. However, peptide retention in RP-HPLC does not always follow this regular behaviour and we now know that in many cases with these ionogenic solutes, the retention process is much more complex than simple hydrophobic expulsion of peptidic solutes from a mixed aqueous–organic solvent eluent. Anomalous retention behaviour for peptides separated on alkylsilicas, has been discussed[1-4] in terms of secondary chemical equilibria, notably solvation effects and ion-pairing phenomena. Most retention irregularities can be ascribed to peptide interaction with accessible silanol groups at the surface of the stationary phase. The heterogeneous nature of the surface of octyl- and octadecyl-silica stationary phases is well documented. Broad and asymmetric peaks have been observed[22-25] when amino compounds and related cationic solutes have been chromatographed on alkylsilica supports under certain conditions. In addition, plots of ln k' versus ψ_s for such compounds may show minima[3,26,27]. Horváth and coworkers[15,16] recently evaluated retention of polar solutes on bonded stationary phases in terms of a dual solvophobic–silanophilic retention mechanism. Detailed investigations with cationic solutes, including protonated peptides, have led other workers to propose[1-3,23] similar two site adsorption models with the surface silanol groups being implicated in peak tailing and regional selectivity changes. With this previous information in mind, we have investigated the retention behaviour of three series of peptides (Table I) using hydro-organic eluents containing different concentrations of methanol, acetonitrile or propan-2-ol.

The results obtained with the phenylalanine oligomers on a μBondapak/C$_{18}$ column with aqueous methanol, acetonitrile or propan-2-ol that contained 20 mM orthophosphoric acid, pH 2.25, are depicted in Fig. 1A, C and E. Shown in Fig. 1B, D and F are the results obtained under similar solvent compositions with mobile phases containing 15 mM triethylammonium phosphate, pH 2.95. For all cases it can be seen that plots of ln k' versus ψ_s are non-linear, and when acetonitrile or methanol was the organic modifier, the plots of ln k' versus ψ_s passed through minima. Further exami-
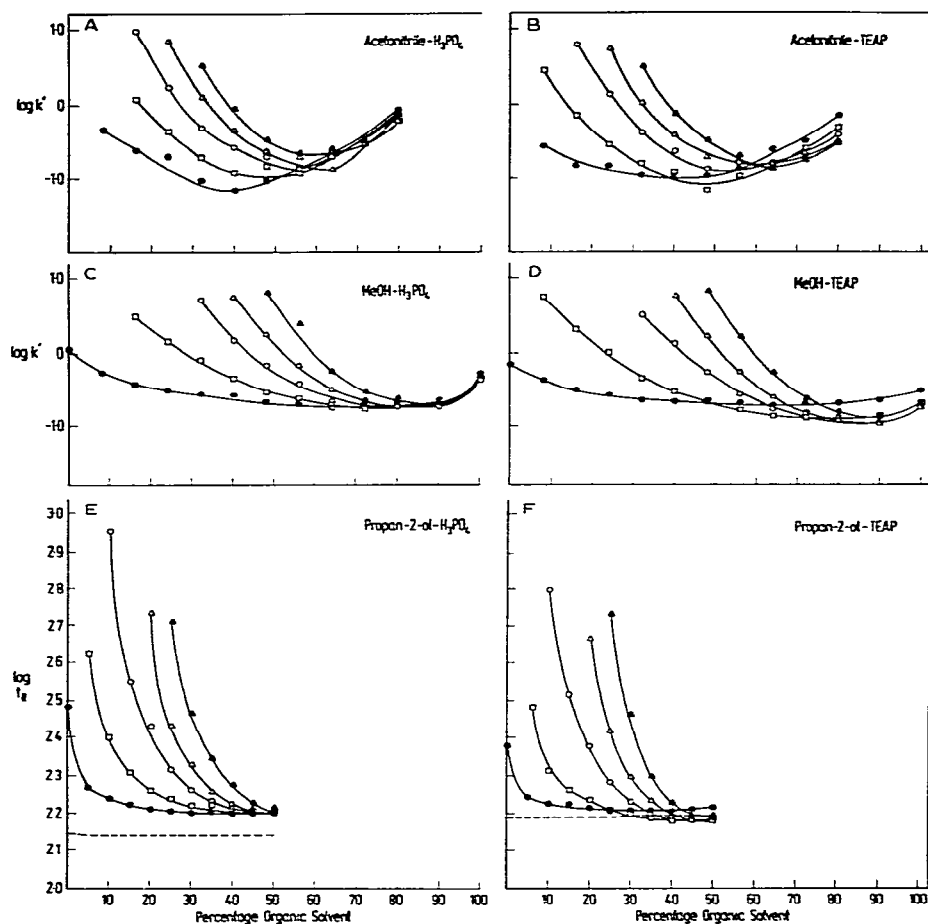
Fig. 1. Plots of the logarithmic retention factors of several phenylalanine oligomers (1–5) against the volume fraction of organic solvent in the aquo-organic solvent mixtures used as the eluent. In A, C and E are shown results obtained with the organic solvent modifiers acetonitrile, methanol or propan-2-ol, respectively with a primary mobile phase comprised of water–20 mM orthophosphoric acid. In B, D and F are shown the data for the corresponding experiments using a primary mobile phase of water–15 mM triethylammonium phosphate. The dashed lines in E and F represent the logarithmic retention time of a sodium nitrate peak. All the data for the propan-2-ol experiments are expressed in terms of retention times (sec). Column, μBondapak C_{18}; flow-rate, 2.0 ml/min for the acetonitrile and methanol experiments, 1.2 ml/min for the propan-2-ol experiments. The phenylalanine oligomer key is: ● = F; □ = FF: ○ = FFF; △ = FFFF; ▲ = FFFFF.

nation of the data reveals several important features germane to the role of the organic solvent modifier. Firstly, it can be readily seen that the capacity factors for the phenylalanine oligomers initially show progressive decreases with water rich eluents as ψ_s is increased. For all three solvents under conditions where $\psi_s < 0.4$, the peptide elution order follows the anticipated regular hydrophobic retention behaviour. Variation in the capacity factors for the different phenylalanine oligomers on changing from methanol to acetonitrile or propan-2-ol are in accord with differences in their respective solvent strengths. However, as ψ_s is increased further

above *ca.* 0.4 a progressive reversal in elution order becomes evident. Finally with water-lean or neat organic solvent eluents, *e.g.* $0.8 < \psi_s < 1$, these phenylalanine oligomers elute in an order characteristic of a normal phase separation mode. This data suggests that the interaction of peptides with alkylsilicas involves both a hydrophobic and a silanophilic component in the retention mechanism. The results obtained with the same eluents containing 15 mM triethylammonium phosphate are thus of special interest. The incorporation of aliphatic amines in the mobile phase has been used to mask peak tailing of cationic solutes[1–3,16,23–25,28–30]. Appropriate concentrations of acidic amine buffers to ensure that silanol effects are negligible can be estimated from column efficiency or binding constant measurements. For peptide separations on alkylsilicas of low to intermediate carbon coverage, triethylammonium salts at concentrations ranging between 10–150 mM have been recommended[1,2,6,29] to improve peak shape and reduce retention. Stein and coworkers[31,32] have favoured 0.5–1 M pyridinium salts although under these conditions, post column fluorometric derivatisation and detection of the eluted peptides are required. With high coverage alkylsilica supports, the retention of basic peptides has been found[1,25] to be practically independent of sample load when *ca.* 2 mM triethylammonium, and related trialkylammonium, buffers are added to the mobile phase. With bulky, amphiphatic alkylamines such as dodecylamine or N,N-dimethyldodecylamine, concentrations as low as 1 mM have been effective[16,24] Although a major effect of acidic alkylamine buffers is thought to involve the masking of accessible polar groups on the surface of the stationary phase, the ability of these buffer components to specifically modify the support into a dynamically coated weak anion exchanger must not be overlooked. Evidence for such an additional role has been presented previously[33–36] and may explain several observations made in the present study, particularly with eluents containing propan-2-ol. For example, with the 15 mM triethylammonium phosphate–propan-2-ol mobile phases, the t_R of the nitrate anion, which was used to calibrate the column dead volume, was larger than the t_R of several of the protonated peptides. Furthermore, the t_R (nitrate) value was concentration dependent. For these reasons, the data for the propan-2-ol experiments are expressed as plots of ln t_R (observed) *versus* ψ_s. Several other small anions, including formate, and acetate, also exhibited enhanced t_R values under these 15 mM triethylammonium phosphate–propan-2-ol elution conditions. The role of such dynamic ion-exclusion and ion-exchange phenomena in the RP-HPLC of peptides with such eluents is, at this stage, poorly delineated and clearly warrants further attention. Evidence in favour of organic amines being extracted onto the stationary phase from the mobile phase appears however to be overwhelming.

Several studies have demonstrated[12,18,36,37] that the organic solvent in a hydro-organic solvent eluent is also distributed between the mobile and stationary phases in RP-HPLC. Determination of the Langmuir adsorption isotherms of a number of organic solvent–water systems in contact with hydrocarbonaceous phases has revealed[12,36] that a layer of solvent molecules form at the bonded-phase surface. The extent of this layer increases with solvent concentration and is inversely related to the dielectric constant, ε, and the elutropic value ε_0 (alumina), of the pure solvent. Specific adsorption of solvent molecules by the stationary phase will give rise to solvent-dependent changes in the surface characteristics of the non-polar support. As is evident from the data shown in Figs. 1–3 these effects have important sequelae as
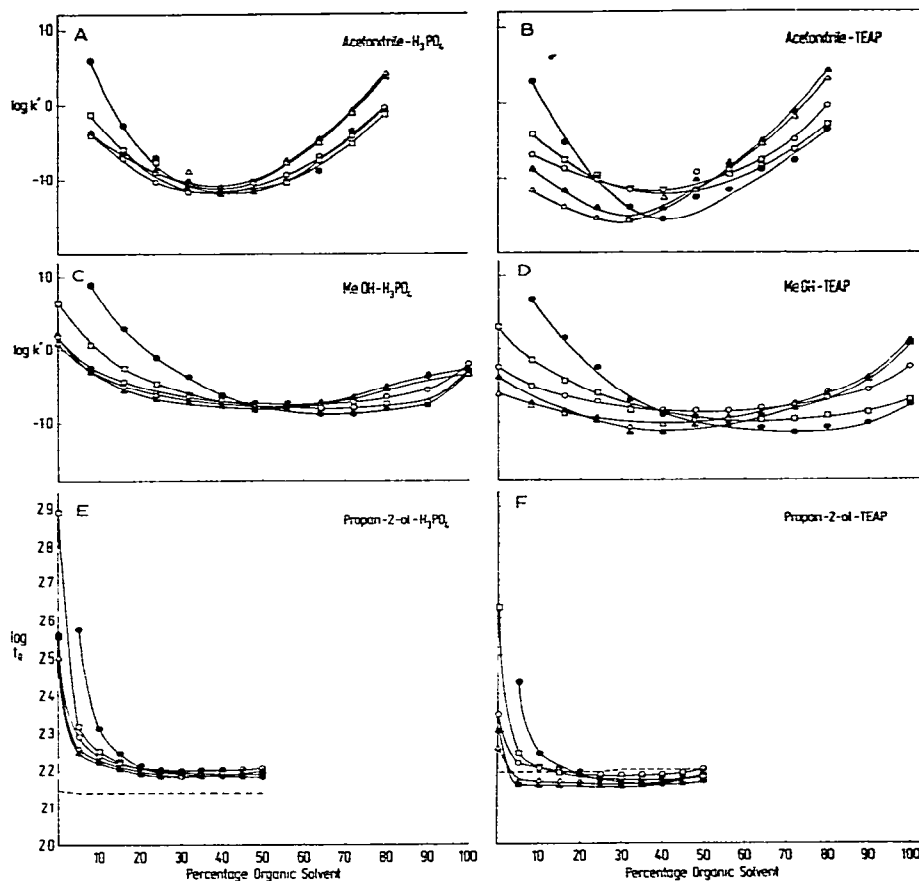
Fig. 2. Plots of the logarithmic retention factors for the peptides (6–10) against the volume fraction of the organic solvent in the water–organic solvent mixture used as the eluent. The chromatographic conditions are the same as in the legend to Fig. 1. The peptides key is: ● = YYY; □ = LY; ○ = PY; △ = RF; ▲ = RFA.

far as the hydrogen bonding processes which occur between peptide solutes and the stationary phase.

Peptides with ionogenic side chain groups also exhibit the concave binodal dependence of ln k' on ψ_s (Figs. 2 and 3). With water-rich eluents, the basic peptides tended to show lower retention than neutral or acidic peptides. However, at the other mobile phase extreme, the opposite pattern was generally seen. These observations, again, are compatible with dual or multiple retention processes being involved in the binding of the peptides to the stationary phase. In common with earlier observations[1,2], peptide retention with mobile phases where $\psi_s < 0.4$ appears independent of the number of amino acid residues. Selectivity was, however, responsive to pH changes which influence the extent of ionisation. It is noteworthy that the curvature of the ln k' dependence on ψ_s for the different small peptides examined in this study varied considerably between the three organic solvents. Many of the trends can be rationalised on the basis of specific solute interactions with the extracted modifier in the
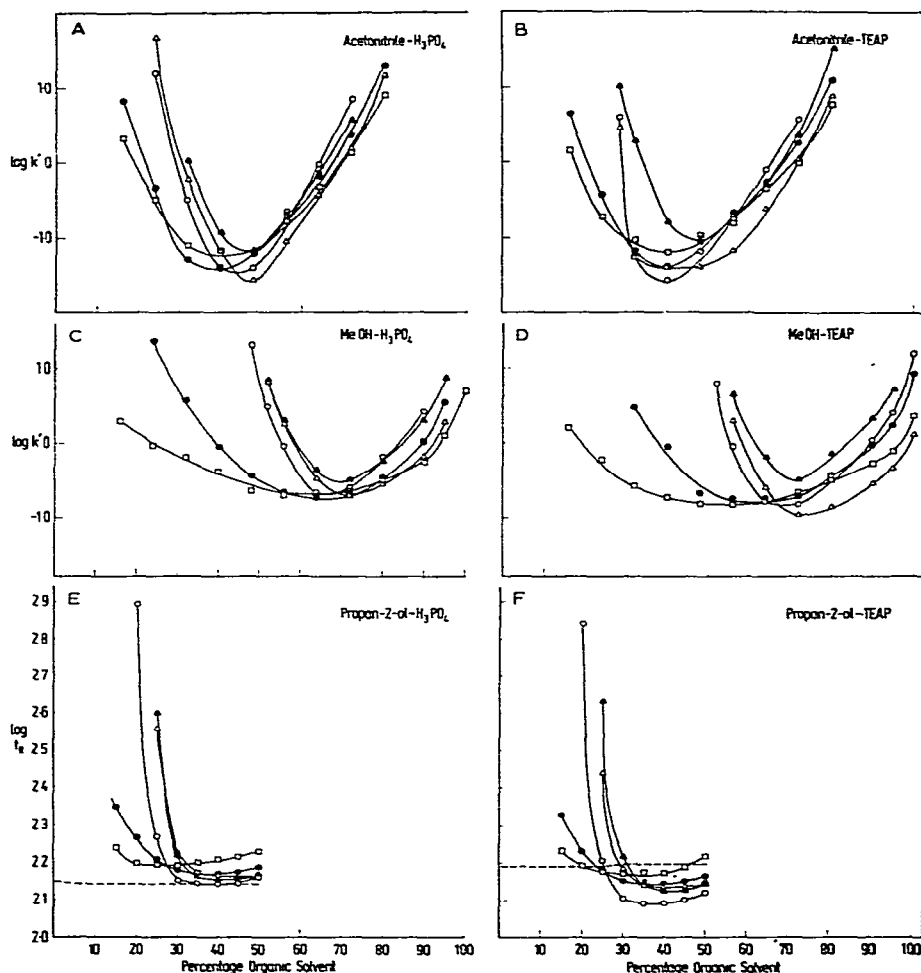
Fig. 3. Graphs illustrating the relationships of the retention factors of several polypeptide hormones (11–15) on octadecylsilica and the composition of the aquo-organic eluent. The chromatographic conditions were the same as given in the legend to Fig. 1. The polypeptides key is: ● = angiotensin I; □ = angtiotensin II; O = bovine insulin; △ = bovine insulin B chain; ▲ = porcine glucagon.

stationary phase. Whereas methanol and 2-propanol are proton accepting solvents, *i.e.* in terms of Pearson's classification[38] these solvents are hard bases, acetonitrile is a weak dipolar base. One consequence of these solvent differences is their ability to manipulate the hydrogen bonding characteristics of the stationary phase. Such variations reflected in the dependence of ln k' on ψ_s could thus account for regional selectivity changes occasionally found when one solvent is substituted by another.

During these studies it was also apparent that column efficiencies were strongly influenced by alterations in the mobile phase composition. Broader peaks were generally observed for the smaller peptides using the more viscous propan-2-ol mobile phases than with the methanol- or acetonitrile-based eluents under otherwise identical conditions. Similar changes in theoretical plate numbers (*N*) for a given column

with increasing eluent viscosity have been noted[17,39] previously in ion-pair RP-HPLC studies of simple amines and small peptides. Fig. 4 shows the influence of flow-rate on column efficiencies for several polypeptide hormones. The mobile phase compositions were chosen to give comparable capacity factors for these polypeptides eluted under isocratic conditions. In all cases a reduction in flow-rate improved the chromatographic efficiencies, with larger changes evident for the more hydrophobic polypeptides. When similar separations were carried out under gradient eluent conditions, the apparent plate counts were *ca.* 50–100 fold larger, that is the apparent heights equivalent to a theoretical plate (HETPs) were *ca.* 0.01 mm.
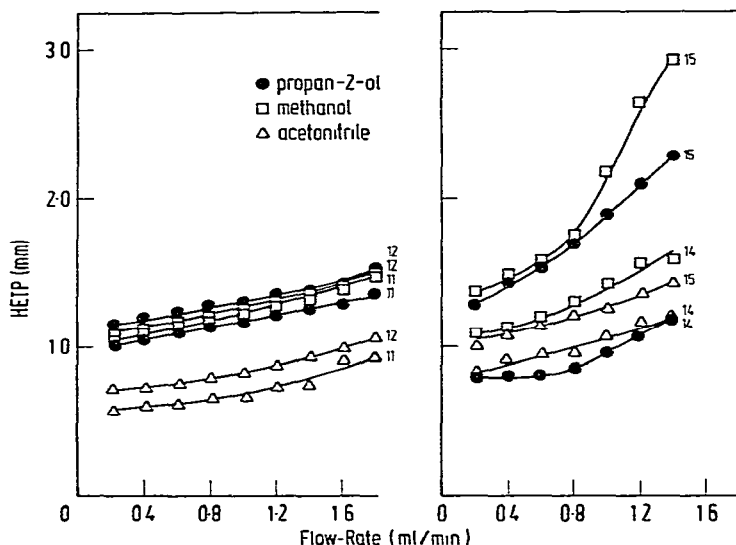

Fig. 4. Effects of mobile phase flow-rate and organic solvent composition on column efficiency for several polypeptides eluted under isocratic conditions. The aqueous 20 mM orthophosphoric acid mobile phases contained different percentages of the three organic solvents (acetonitrile, methanol and propan-2-ol) such that the polypeptides eluted with comparable capacity factors. The peptide key is: 11, angiotensin II; 12, angiotensin I; 14, bovine insulin; 15, porcine glucagon.

Knowledge of the dependence of ln k' on ψ_s has important consequences for the isolation of peptides and polypeptides from tissue sources. Since a given capacity factor can be obtained at two different eluent compositions, it should be possible to choose one organic solvent percentage which permits optimal resolution of a particular component. Although silanol effects are generally considered deleterious in peptide separation by RP-HPLC techniques, under appropriate conditions they permit additional control over selectivity. As we have demonstrated elsewhere[1,6], silanol effects may in fact prove advantageous for the preparative separation of hydrophobic polypeptides and proteins, including several pituitary protein hormones. Similar conclusions have been reached by Bij *et al.*[16] from their studies on hydrophobic peptide fragments of glycophorin A. A very steep dependence of ln k' on ψ_s would imply that very little elution development occurs for a particular peptide–polypeptide during the separation. Although superficially, this could be considered undesirable, in reality such a dependence may be quite useful in obtaining adequate

selectivity for a separation of biological peptides, many of which are available in amounts less than 1 μg per g tissues. For example, the nature of the dependence of ln k' on ψ_s for peptides and polypeptides permits the reversed-phase support to be used for the concentration of peptides from very dilute solutions such as extracts or haemodialysates[40]. Our experience has indicated that linear gradients for acetonitrile ($0 < \psi_s < 0.5$), and shallow convex gradients for methanol ($0 < \psi_s < 0.7$) and 2-propanol ($0 < \psi_s < 0.4$) are optimal gradient configurations for polypeptide resolution on surface modified hydrocarbonaceous silicas. Comparison of apparent column efficiencies reaffirms the value of gradient elution particularly in the initial stage of a micro or small scale RP-HPLC fractionation of a given component from a complex biological mixture. Batch elution methods using a window of organic solvent percentages chosen from ln k' versus ψ_s data, however, are currently more convenient for large scale preparative separations of peptides involving more than 1-g samples[41]. The use of ternary mobile phases provides an alternative approach to enhance polar selectivity effects and modulate retention. In this context low concentrations of a second organic solvent modifier, for example 1 % tert.-propanol or trifluoroethanol, have proved[1,25] effective for the modulation of peptide retention to hydrocarbonaceous bonded silicas.

In conclusion, over a wide range of ψ_s values, minima are observed in the plots of ln k' versus ψ_s for peptides separated on octadecylsilica. With mobile phases of low water content, the elution order of unprotected peptides follows a normal-phase pattern. The curvature of the bimodal plots is influenced by the hydrophobicity of the peptide and the characteristics of the organic solvents. In accord with earlier observations[1-4], with water-rich eluents encompassing a narrow ψ_s range the regular retention behaviour exhibited by peptides can be approximated to linear dependencies of ln k' on ψ_s. Evaluation of the overall dependence of ln k' on ψ_s permits mobile phase compositions to be selected allowing optimal resolution of peptides on alkylsilica supports. Similar RP-HPLC methods can be extended to the separation and recovery of undegraded proteins. For example, on 50-nm pore diameter octylsilicas we have been able to resolve[1,42] ovine thyrotrophins and human thyroglobulin 19S iodoproteins including the desialo-19S iodoprotein with preservation of native biological activity.

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